

## 9

# Supersensitive *In Situ* Hybridization by Tyramide Signal Amplification and Nanogold<sup>®</sup> Silver Staining: The Contribution of Autometallography and Catalyzed Reporter Deposition to the Rejuvenation of *In Situ* Hybridization

Raymond R. Tubbs, James Pettay, Thomas Grogan,  
Annie L.M. Cheung, Richard D. Powell, James Hainfeld,  
Cornelia Hauser-Kronberger, and Gerhard W. Hacker

## INTRODUCTION

It is peculiar that *in situ* hybridization (ISH), a technique with many similarities to immunohistochemistry (IHC), has not enjoyed the phenomenal growth in both basic research and clinical applications as has its sister technique IHC. Since the late 1970s, when immunoperoxidase techniques began to be applied to routine diagnostic material and to numerous research applications, there has been a natural evolution of the IHC procedure. Namely, only a few primary antibodies were available commercially at the onset, and only one indirect and the peroxidase-antiperoxidase

(PAP) technique detection systems were in place. With the advent of avidin-biotin detection systems and monoclonal antibodies, and a viable commercial market, extraordinary growth of the procedure's applications in clinical research and diagnostic pathology occurred during the subsequent two decades. Today, IHC is automated and widely used for research purposes and, to a large extent, has become a routine diagnostic "special stain" in most clinical laboratories.

During the same period, ISH enjoyed very little growth in both research and diagnostic applications. What has accounted for this lack of maturation of the technique?

## Gold and Silver Staining

The success of IHC is part of the reason measuring a gene's encoded protein routinely and inexpensively, particularly as automation evolved, rendered IHC a more viable choice in many instances. Inherent comparative sensitivity of the procedures has also clearly been a factor. Unfortunately, the chromogenic procedures in place are often insufficiently sensitive to detect the relatively low amounts of DNA and RNA levels at which the clinical utility is to be found.

But ISH is enjoying a renaissance, as reflected in Protocols 1 through 6 and Figures 9.1 through 9.4. There are multiple reasons for this rejuvenation. First, great advances have been achieved through enhanced reporter systems. Detection systems, now of either fluorescence, chromogenic, or autometallographic type, can utilize variations of tyramide signal amplification (TSA).<sup>2,8,10,14-16,19,20,25</sup> Also known as catalyzed reporter deposition (CARD), TSA exploits the catalytic action of peroxidase on tyramide conjugates. Single copies of the human papillomavirus (HPV) can now be routinely detected in human cells with this technology.<sup>25</sup> Advances in cell conditioning have also greatly enhanced the practical sensitivity of ISH, and when used rationally in combination with TSA, tremendous analytical strength is achieved. The detection of individual or amplified endogenous genes such as the oncogene *Her-2/neu*, with proper cell conditioning and signal amplification, can be done by either fluorescent or bright-field methods. Autometallography itself also provides for enhanced signal intensity with clean background, particularly when Nanogold® covalent particles are used.<sup>4-13,17,18,21,22</sup> Perhaps the greatest boon to the renaissance of ISH will be the identification of truly remarkable new applications for the technology, such as routine bright-field *in situ* target detection with morphologic correlation, or unique combinations of fluorescence, autometallography, and

chromogenic preparations.<sup>24</sup>

With the advent of automation, bright-field ISH applications can potentially be combined with IHC, whereby endogenous and the gene's encoded protein can be simultaneously visualized. One such technique has recently been developed (see Protocol 5 and Figure 9.3) and was named CODFISH (concomitant oncoprotein detection with fluorescence *in situ* hybridization [FISH]). Autometallography and subtracted unique sequence yeast artificial chromosome (YAC) probe-based ISH, may also potentially be combined with IHC.

Finally, given the explosive growth of knowledge of the human genome, large-scale screening via tissue microarrays and the advent of new probe technologies such

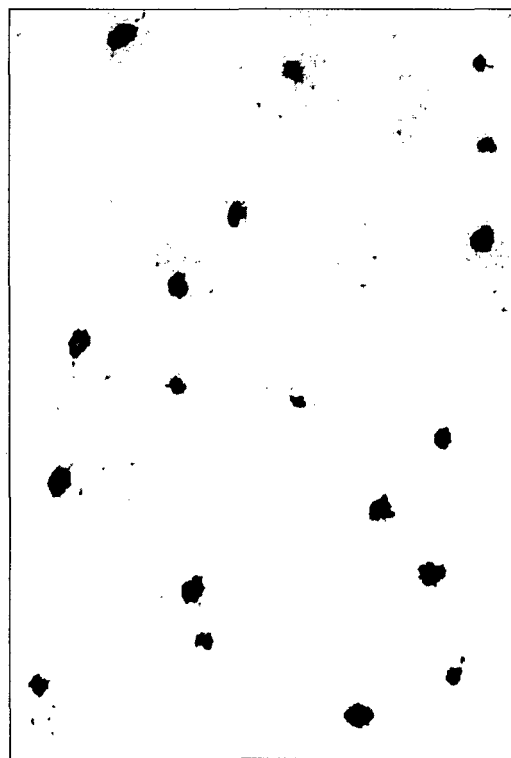


Figure 9.1. Squamous cell carcinoma of the uterine cervix with genome-integrated HPV 16/18 copies. Nearly every tumor cell nucleus shows one black dot, very likely representing a single HPV 16/18 copy detected as in Protocol 2.

as subtractive unique sequence ISH probes derived from bacterial artificial chromosomes (BACs) or YACs dramatically augment signal intensity by virtue of probe size, sequence specificity, and subtraction of cross-hybridization polymorphic loci.<sup>1,3</sup> Even chromosomal deletions can be profiled in interphase FISH or chromogenic *in situ* hybridization (CISH) format, thereby opening even greater vistas for the revised and rejuvenated ISH procedure.

### REAGENTS, SUPPLIES, AND INSTRUMENTATION

The majority of reagents necessary to perform ultrasensitive autometallographic ISH are available commercially. In fact, patents are held for some of the key components of the technology, precluding at least in house "home brew" format synthe-

sis of the reagents, at least if clinical applications are anticipated.

Reagents for autometallography are available from Nanoprobes (Yaphank, NY, USA) (<http://www.nanoprobes.com/>), and it is also possible to use alternative silver enhancers such as silver acetate autometallography.<sup>9</sup> For procedures that depend upon tyramide-based amplification, the basic patent is owned by NEN Life Science Products (Boston, MA, USA) as the renaissance kit. Manufacturers such as DAKO (Carpinteria, CA, USA) have adapted the tyramide technology to a stable kit format for detection of DNA targets, which is available as the GenPoint® kit. All the other reagents are available from standard chemical manufacturers and are mainstays of the typical molecular pathology laboratory. These include phosphate-buffered saline (PBS), standard sodium citrate buffer (SSC), graded alcohols,

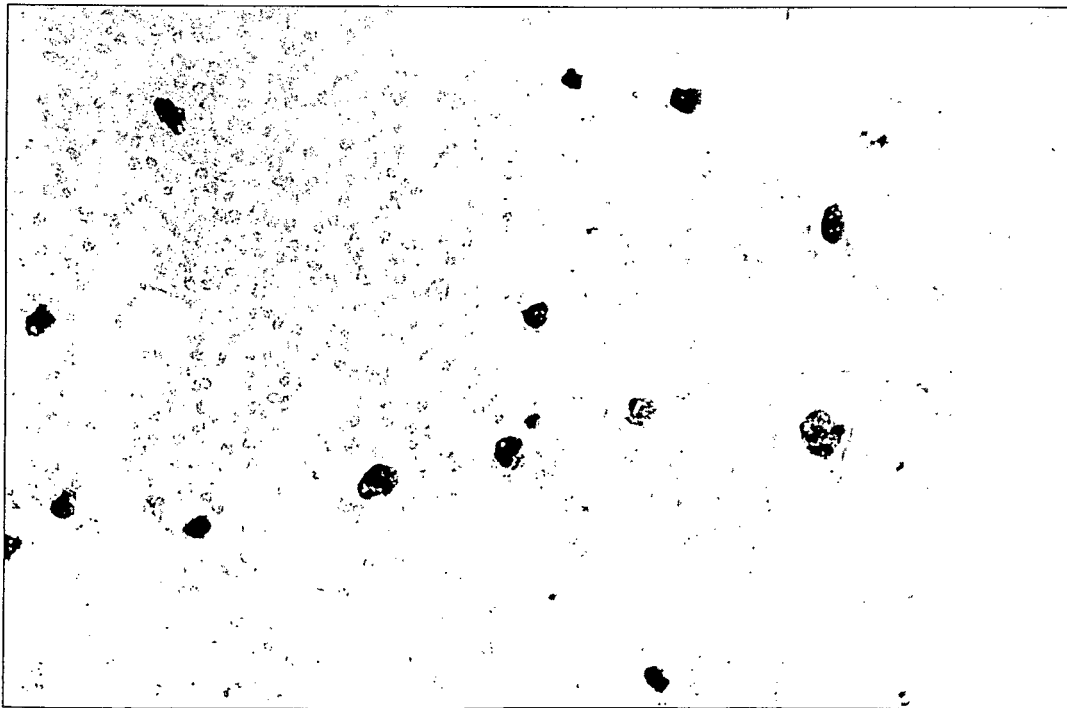


Figure 9.2. Hodgkin lymphoma mononuclear cell variants demonstrating Epstein-Barr Virus associated RNA (EBER). Cells containing EBER RNA of EBV are labeled in black, using the procedure described in Protocol 3.

## Gold and Silver Staining

organic solvents, and similar reagents.

A variety of cell conditioning approaches have been advocated in the literature. In general, these approaches utilize heat and a variety of buffer solutions or chelating agents. Steamers, pressure cookers, water baths, and microwave treatments have all been advocated. As a general rule, each ISH system will usually have its own peculiar requirements for cell conditioning (heated epitope retrieval, antigen recovery, etc.), and empirical approaches using combinations of buffers and duration of heat are usually necessary. More recently, an automated instrument has become available (Discovery® and Benchmark®; Ventana Medical Systems, Tucson, AZ, USA) for which cell conditioning prior to ISH are performed on-line by the instrument. This approach includes removal of paraffin and cell conditioning in the presence of proprietary buffers. Consistency of use of this instrument should have a very beneficial effect on reproducible performance of ISH techniques.

Probes for ISH may consist of genomic DNA probes, ribroprobes, or oligonucleotide probes. Many excellent reagents are available from commercial sources, but much of what is currently performed is based upon home brew probe reagents for both clinical research and some clinical applications. Whether of commercial or home brew origin, the performance of each probe, and the detection system utilized, must be carefully validated. Guidelines for such validation have been previously published.

Multiple detection systems for fluorescent and chromogenic–autometallographic assays are available. For example, direct conjugates for FISH analysis, including up to 4-color direct conjugate systems, are available from Vysis (Downers Grove, IL, USA). Some preliminary work has been done in the commercial sector to develop chromogenic ISH probes, and these are available commercially from several sources (DAKO,

Zymed Laboratories [South San Francisco, CA, USA], Ventana Medical Systems, Vector Laboratories [Burlingame, CA, USA], Research Genetics [Huntsville, AL, USA], Enzo Diagnostics [Farmingdale, NY, USA], and others). Once again, it must be emphasized that each probe, and the detection system used for that system, must be carefully validated using published guidelines.

Stability of biotin–tyramide conjugates is a serious issue, but has largely been achieved by its two manufacturers, NEN Life Science Products and DAKO. In particular, the DAKO GenPoint kit has achieved a remarkably stable tyramide reagent supported by that system, and in combination with ultrapure streptavidin–peroxidase, provides for excellent and reproducible results. Other immunogenic compounds can be labeled with tyramide including fluorescein isothio-



Figure 9.3. (See color insert Figure 9.3 following page 78.) Fluorescence confocal photomicrograph of CODFISH preparation. Paraffin section of cell line known to display amplification of the *Her-2/neu* gene (green) and its encoded protein (red). Amplification–overexpression is identified (amplified gene copy is green, increased *Her-2/neu* oncoprotein is red).

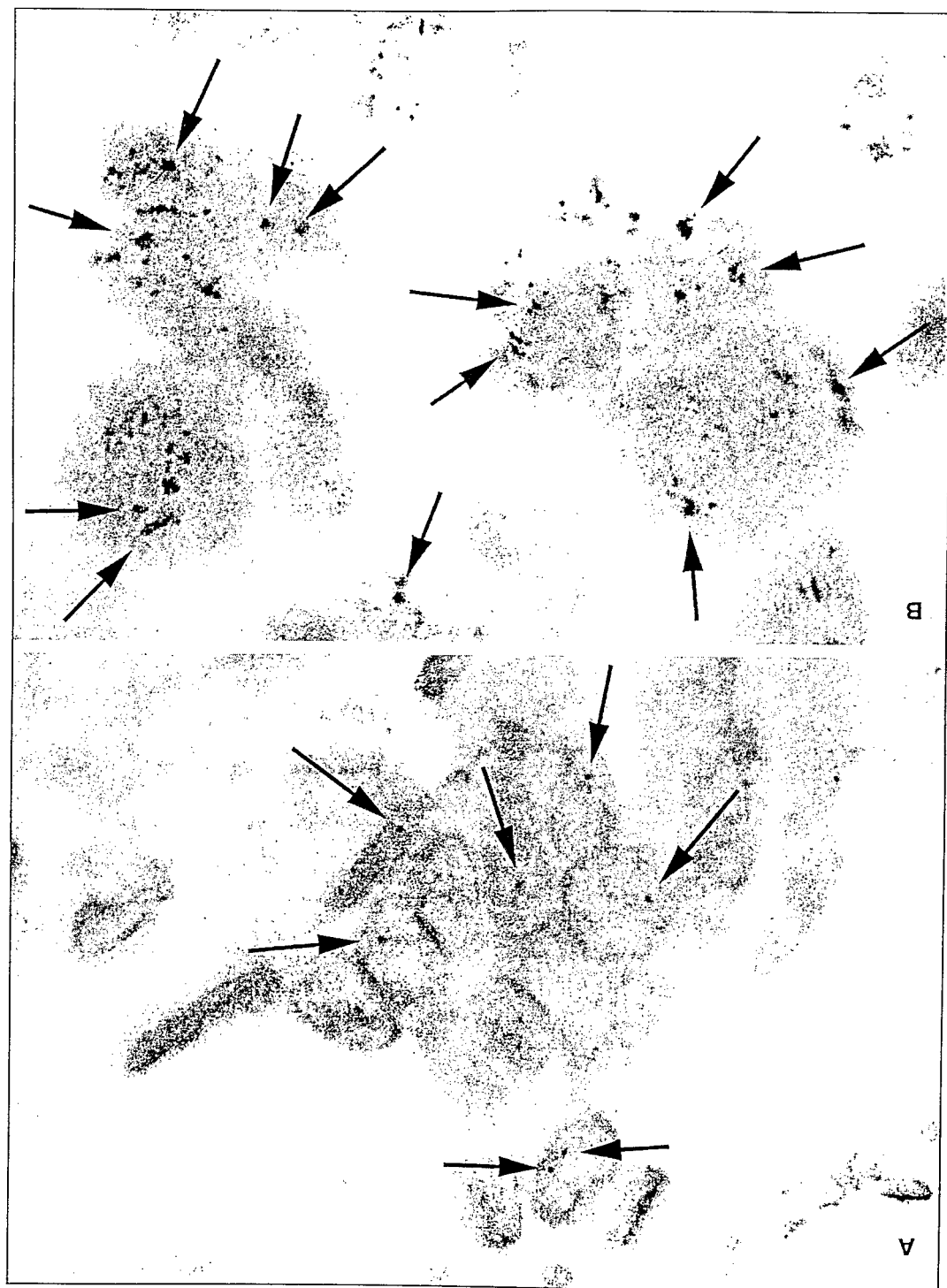


Figure 9.4. GOLDSULFIDE preparations. Autometallography using subtractive unique sequence ISH identifies 1 or 2 copies of *Her-2/neu* in a subpopulation of normal truncated nuclei (A; arrows) and overexpression-unique sequence ISH identification in an invasive ductal carcinoma (B).

## Gold and Silver Staining

cyanate (FITC), digoxigenin, and dinitrophenol (DNP). Also, the nucleotides can be directly labeled with peroxidase. Several commercial sources will provide custom conjugation services for these haptens.

### PROTOCOLS

**Note:** The protocols included in this chapter undergo frequent refinement, and new similar techniques are added at the following web site: (<http://www.sbg.ac.at/kgg/protocols/protocols.htm>).

#### Protocol 1. DNA *In Situ* Hybridization with Streptavidin–Nanogold

##### *Background and Purpose*

This protocol allows a reliable and very sensitive detection of a few copies of HPV, cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV), and other DNA viruses in routinely formalin-fixed and paraffin-embedded tissues. The method described below has great potential as a robust and fast routine method for diagnostic purposes in all cases where conventional ISH is not sensitive enough, and where actual single-copy sensitivity is not needed. The protocol also works well with FluoroNanogold™ (Nanoprobes), thus allowing visualization of the very same preparation by fluorescent microscopy (FISH) and transmitted light microscopy. Nanogold preparations can be transferred to the electron microscope.<sup>21</sup> Nanogold was developed by Dr. James F. Hainfeld<sup>17</sup> and is available from and patented by Nanoprobes.

##### *Solutions*

- PBS: 10× PBS ( $Mg^{2+}$ - and  $Ca^{2+}$ -free), pH 7.6: 11.36 g  $Na_2HPO_4$ , 2.72 g

$KH_2PO_4$ , 87.0 g NaCl in 800 mL distilled water. Adjust pH with concentrated NaOH and add distilled water to a final volume of 1 L.

- SSC: 175.32 g NaCl and 88.23 g sodium citrate in 800 mL distilled water. Adjust pH with NaOH to 7.0 and add distilled water to a final volume of 1 L.

##### *Procedure*

1. Deparaffinize sections from formaldehyde-fixed tissue in fresh xylene (2 times for 15 min each).
2. Rinse and rehydrate in graded alcohols and distilled water (2–3 min each).
3. Soak in PBS (20 mM, pH 7.6) for 3 min.
4. Incubate with 0.1 mg/mL proteinase K (Code No. 1373196; Roche Molecular Biochemicals, Mannheim, Germany) in PBS at 37°C for about 8 min. The duration is critical and has to be tested very carefully, depending on tissue, fixation, and other factors.
5. Rinse in 2 changes of PBS for 3 min.
6. Permeabilize with 0.3% Triton® X-100 in PBS for 15 min.
7. Wash in PBS for 2 min.
8. Rinse in 2 changes of distilled water, dehydrate with graded alcohols (50%, 70%, and 98% isopropanol) for 1 min each and air-dry the sections.
9. Prehybridize with 1:1 mixture of deionized formamide and 20% dextran sulfate in 2× SSC at 50°C for 5 min.
10. Carefully shake off excess prehybridization block.
11. Add one drop of biotinylated DNA probe on the section and cover with a small coverslip. Avoid air bubbles.
12. Heat sections on heating block at 92° to 94°C for 8 to 10 min to denature DNA.
13. Incubate in a moist chamber at 37°C

- overnight (or for at least 2 h).
  14. Posthybridization washes (5 min each): 2 changes of 4× SSC (first wash to remove coverslips), 2× SSC, 0.1× SSC, 0.05× SSC, and then distilled water.
  15. Put slides into Lugol's iodine solution (Merck, Darmstadt, Germany) for 5 min.
  16. Wash in tap water and then distilled water.
  17. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless. Then wash in tap water for 5 min and distilled water for 2 min.
  18. Immerse in PBS containing 0.1% fish gelatin (45% concentrate; Cat. No. G-7765; Sigma-Aldrich, Steinheim, Germany) and 0.1% Tween® 20 for 5 min.
  19. Incubate sections with streptavidin-Nanogold (Nanoprobes) diluted 1:200 to 1:500 in PBS containing 1% bovine serum albumin (BSA) at room temperature for 60 min.
  20. Wash in 3 changes of PBS containing 0.1% fish gelatin and 0.1% Tween 20 for 5 min each.
  21. Repeatedly wash in distilled water for at least 10 min altogether, the last 2 rinses in ultrapure water (electron microscopy [EM]-grade).
  22. Perform silver acetate autometallography or GoldEnhance® development (Nanoprobes). The procedure for silver acetate is given here:
    - a. Solutions A and B should be freshly prepared for every run. Solution A: dissolve 80 mg silver acetate (Code No. 85140; Fluka Chemical, Buchs, Switzerland) in 40 mL of glass double-distilled water. Silver acetate crystals can be dissolved by continuous stirring within about 15 min.
    - b. Citrate buffer: dissolve 23.5 g of trisodium citrate dihydrate and 25.5 citric acid monohydrate in 850 mL or deionized or distilled water. This buffer can be kept at 4°C for at least 2 to 3 weeks. Before use, adjust to pH 3.8 with citric acid solution.
    - c. Solution B: dissolve 200 mg hydroquinone in 40 mL citrate buffer.
    - d. Enhancement solution: just before use, mix solution A with solution B.
    - e. Silver amplification: place the slides vertically in a glass container (preferably with about 80 mL volume and up to 19 slides; Schieffeder-type) and cover them with the mixture of solutions A and B. Staining intensity can be checked in the light microscope during the amplification process, which usually takes about 5 to 20 min depending on primary antibody or nucleic acid probe concentration, incubation conditions, and the amount of accessible antigen or nucleic acid sequence in question.
    - f. Photographic fixer has been used in combination with colloidal gold to stop the enhancement process immediately. Alternatively, a 2.5% aqueous solution of sodium thiosulfate can be used.
- Urgent:** It has, however, turned out that both these fixing treatments can be harmful when working with labeled tyramides or with Nanogold. We therefore now recommend to stop enhancement simply by washing in distilled water.
- g. After stopping the enhancement process, slides can be examined in a light microscope more carefully. If staining intensity is still too low, wash slides for one more time in double-distilled water and develop further in enhancement solution.

- h. After washing in distilled water, sections can be counterstained with nuclear fast red, or hematoxylin, and/or eosin.
- i. After washing, dehydration in graded alcohols, and clearing in xylene, Permount™ (Fisher Scientific, Pittsburgh, PA, USA) are the preferred mounting media (see companion chapters in this book, especially Chapter 1).

### Protocol 2: DNA *In Situ* Hybridization with Labeled Tyramides and Streptavidin–Nanogold

#### *Background and Purpose*

This method is based on the superb properties of Nanogold and of the catalyzed reporter deposition–tyramide signal amplification system. It allows a supersensitive and relatively reliable detection of even single copies of HPV in routinely formaldehyde-fixed and paraffin-embedded tissue specimens, as well as on formalin-fixed cytological preparations. Other DNA viruses, as well as mRNA and other RNA stainings, have been tested too (see Protocol 3). The protocol given here was developed for formalin-fixed paraffin sections glued onto silanized glass slides. Cytocentrifuge preparations can also be used with the following procedure, but steps 1 and 2 should be eliminated (start at step 3). Cytospins should be air-dried prior to fixation in either neutral-buffered formalin or absolute ethanol.

It must be mentioned that this method is not yet completely problem-free. We experienced repeatedly that the outcome strongly depends on the individual tissue and fixation conditions applied, as well as on the quality and type of hybridization probe used. As the method is extremely sensitive, this also means that even minor

tissue fixation problems or minor probe and reagent quality differences yield massive background staining, thereby sometimes masking structures to be diagnosed. Quite often, intra-nuclear staining of fibroblasts has also been noted. The protocol is therefore given as a guideline for one's own experiments. Concerning reporter molecules, biotin-labeled cDNA probes (Enzo Diagnostics) often gave excellent results when demonstrating HPVs. The quality of the probe for hybridization is of enormous relevance here; small impurities can lead to massive background staining. Riboprobes labeled with FITC have also given good staining in our system (see Protocol 3). It has to be mentioned that we were not yet successful to detect digoxigenin-labeled probes with the tyramide system; one possible explanation may be steric hindrance.

#### *Solutions*

CARD, developed by Dr. Mark Bobrow, is patented by Perkin Elmer Life Sciences under the term TSA™ (<http://www.lifesciences.perkinelmer.com>). We have successfully used biotinylated tyramides (BTs) from the TSA-Indirect kit for ISH. A licensed tyramide product that worked well in our tests is available from DAKO, contained in the GenPoint *In Situ* kit (Cat. No. K0620).

#### *Procedure*

1. Deparaffinize sections from formaldehyde-fixed tissue in fresh xylene (2 times for 15 min each).
2. Rinse in absolute ethanol (2 times for 5 min each).
3. Treat with 3% H<sub>2</sub>O<sub>2</sub> in methanol at room temperature for 30 min.
4. Rinse in double-distilled (ultrapure) water for 10 sec and then in PBS for 3 min.



5. Incubate sections with 0.1 mg/mL proteinase K in PBS at 37°C for about 8 min (optimal duration should be tested). This treatment may partly destroy tissue morphology. However, it is necessary to open up binding sites for the probe to reach full sensitivity. Combination with microwave treatment may be feasible.
6. Wash in 2 changes of PBS for 3 min each, then wash with ultrapure water for 10 sec.
7. Dehydrate with graded alcohols (50%, 70%, absolute ethanol) for 5 min each and air-dry the sections.
8. Prehybridize with 1:1 mixture of deionized formamide and 20% dextran sulfate in 2× SSC at 50°C for 5 min.
9. Carefully shake off the excess prehybridization block.
10. Add one drop of biotinylated DNA probe on the section and cover with a small coverslip. Avoid air bubbles.
11. Heat sections on heating block at 92° to 94°C for 8 to 10 min to denature DNA.
12. Incubate in a moist chamber at 37°C overnight (or for at least 2 h).
13. Posthybridization washes (5 min each): 2 changes of 2× SSC (first wash to remove coverslips), 0.5× SSC, 0.2× SSC, and then distilled water.
14. Put slides into Lugol's iodine solution for 5 min.
15. Wash in tap water and then double-distilled water.
16. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless. Then wash in distilled water for 2 min.
17. Drain off section, wipe area around the section dry, and surround it with a DAKO-Pen (Cat. No. S-2002; DAKO).
18. Incubate with blocking solution at 37°C for 30 min. Blocking solution is 4× SSC containing 5% casein sodium salt (Cat. No. C-8654; Sigma) or 0.5% blocking powder (from Renaissance TSA-indirect ISH kit; Cat. No. NEL730; Perkin Elmer Life Sciences).
19. Briefly wash in 4× SSC containing 0.05% Tween 20 for 2 min.
20. Incubate with streptavidin-biotin-peroxidase complex (e.g., StreptABComplex/HRP Duet kit; Cat. No. K0492; DAKO) at room temperature for 30 min. This complex is dissolved in the above blocking solution at a concentration of 1:200.
21. Wash in 3 changes of 4× SSC containing 0.05% Tween 20 for 2 min each, followed by 2 changes of PBS for 2 min each.
22. Incubate the sections with BT at room temperature for exactly 10 (with BT from Renaissance TSA-indirect ISH kit) or 15 min (with BT from GenPoint *In Situ* kit). The BT reagent in the GenPoint kit is ready-to-use. For the TSA-indirect kit, a stock solution of BT is prepared by adding 100 mL ethanol to the lyophilized reagent and is diluted 1/50 to 1/100 with the supplied amplification diluent mixed with distilled water at 1:1, as described in the kit. According to the guidelines supplied, the working solution should contain 1 or 0.5 mg of BT per mL diluent, consisting of 0.2 mol/L Tris-HCl, 10 mmol/L imidazole, pH 8.8, and 0.01% H<sub>2</sub>O<sub>2</sub>.
23. Wash in 4 changes of PBS containing 0.05% Tween 20 and 20% dimethyl sulfoxide (DMSO) at room temperature for 3 min each.
24. Immerse in PBS-gelatin (PBS containing 0.1% fish gelatin) for 5 min.
25. Incubate the sections with streptavidin-Nanogold diluted 1:750 in PBS containing 1% BSA at room temperature for 60 min.
26. Wash in 3 changes of PBS-gelatin for 5

## Gold and Silver Staining

min each.

27. Repeatedly wash in ultrapure water (EM-grade).

28. Perform silver acetate autometallography (see Protocol 1).

**Note:** For all applications where Nanogold is used instead of colloidal gold, it is crucial that slides are not dipped into sodium thiosulfate solution to stop the silver enhancement process. Instead, the development process should be interrupted by simply washing the sections in distilled water (several changes). Sodium thiosulfate would remove the black Nanogold silver-gold staining already obtained.

29. After autometallographic amplification, sections can be counterstained with hematoxylin and eosin and/or nuclear fast red, dehydrated, and mounted in Permount or in DPX.

### Protocol 3. RNA *In Situ* Hybridization with Labeled Tyramides and Streptavidin-Nanogold<sup>7,23</sup>

#### *Practical Considerations*

The same precautions as given in Protocol 2 have to be taken. As the following protocol is the working procedure for RNA, additional guidelines are necessary. RNA is extremely labile and especially susceptible to destruction by RNase. RNA loss from tissue may occur following delays in primary fixation or also because of production of endonucleases by bacteria-contaminated water baths and other equipment used in preparing tissues. In routine histopathological diagnosis, there is little the molecular pathologist can do to control fixation conditions beyond admonition of his or her colleagues and attention to processing protocols. Great care should be

exercised to keep microtome blades and water baths clean and free of bacterial contamination, and sterile water should be used during the hybridization step. Either riboprobes or synthetic oligonucleotides labeled with a reporter molecule such as FITC or biotin may be used. Digoxigenin as the reporter molecule did not work in our tyramide signal-amplified ISH protocols.

Hybridization conditions and nucleotide content must be appropriate for RNA rather than DNA hybridization. The protocol given here for FITC as the reporter molecule was developed for formalin-fixed paraffin sections on silanized glass slides. Cyto centrifuge preparations can also be used with the following procedure, but steps 1 and 2 should be eliminated (start at step 3). Cytospins should be air-dried prior to fixation in either neutral-buffered formalin or absolute ethanol.

#### *Solutions*

- PBS: 10× PBS ( $Mg^{2+}$ - and  $Ca^{2+}$ -free), pH 7.6: 11.36 g  $Na_2HPO_4$ , 2.72 g  $KH_2PO_4$ , 87.0 g NaCl in 800 mL distilled water. Adjust pH with concentrated NaOH and add distilled water to a final volume of 1 L.
- SSC: 175.32 g NaCl and 88.23 g sodium citrate in 800 mL distilled water. Adjust pH with NaOH to 7.0 and add distilled water to a final volume of 1 L.

#### *Procedure*

1. Deparaffinize formaldehyde-fixed sections in fresh xylene (2 times for 15 min each).
2. Rinse in absolute ethanol (2 times for 5 min each).
3. Treat with 3%  $H_2O_2$  in methanol at room temperature for 30 min.
4. Rinse in double-distilled (ultrapure) water for 10 sec and then in PBS for 3 min.

5. Incubate sections with 0.1 mg/mL proteinase K in PBS at 37°C for about 8 min. Optimal concentration and duration is critical and should be tested carefully. This treatment may partly destroy tissue morphology. However, it is necessary to open up binding sites for the probe to reach full sensitivity. Combination with microwave treatment may be feasible.
6. Wash in 2 changes of PBS for 3 min each, then wash with ultrapure water for 10 sec.
7. Dehydrate with graded alcohols (50%, 70%, absolute ethanol) for 5 min each and air-dry the sections.
8. Prehybridize with 1:1 mixture of deionized formamide and 20% dextran sulfate in 2× SSC at 50°C for 5 min.
9. Carefully shake off the excess prehybridization block.
10. Add one drop of FITC haptenated ribonucleotide or antisense oligonucleotide probe on the section and cover with a small coverslip. Avoid air bubbles.
11. Hybridize in a moist chamber at 37°C overnight (or for at least 2 h).
12. Posthybridization washes (5 min each): 2 changes of 2× SSC (first wash to remove coverslips), 0.5× SSC, 0.2× SSC, and then distilled water.
13. Wash in PBS for 3 min.
14. Drain off section, wipe area around the section dry, and surround it with a DAKO-Pen.
15. Incubate with mouse monoclonal anti-FITC antibody (Cat. No. BA-9200; Roche Molecular Biochemicals) diluted in PBS at a working dilution of 1:1000 at room temperature for 30 min.
16. Wash in PBS twice for 3 min each.
17. Incubate with biotinylated goat anti-mouse IgG antibody (Vector Laboratories) diluted in PBS at a working dilution of 1:200 at room temperature for 30 min.
18. Wash in PBS twice for 3 min each.
19. Put slides into Lugol's iodine solution for 5 min.
20. Wash in tap water and then double-distilled water.
21. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless. Then wash in distilled water for 2 min.
22. Incubate with blocking solution at 37°C for 30 min. Blocking solution is 4× SSC containing 5% casein sodium salt or 0.5% blocking powder (from Renaissance TSA-indirect ISH kit).
23. Briefly wash in 4× SSC containing 0.05% Tween 20 for 2 min.
24. Incubate with streptavidin-biotin-peroxidase complex (e.g., from StreptAB-Complex/HRP Duet kit) at room temperature for 30 min. The complex is dissolved in the above blocking solution at a concentration of 1:200.
25. Wash in 3 changes of 4× SSC containing 0.05% Tween 20 for 2 min each, followed by 2 changes of PBS for 2 min each.
26. Incubate the sections with BT at room temperature for exactly 10 (with BT from Renaissance TSA-indirect ISH kit) or 15 min (with BT from Gen-Point *in situ* kit). For the TSA-indirect kit, a stock solution of BT is prepared by adding 100 mL ethanol to the lyophilized reagent and is diluted 1/50 to 1/100 with the supplied amplification diluent mixed with distilled water at 1:1, as described in the kit. According to the guidelines supplied, the working solution should contain 1 or 0.5 mg of BT per mL diluent, consisting of 0.2 M Tris-HCl, 10 mM imidazole, pH 8.8, and 0.01% H<sub>2</sub>O<sub>2</sub>.
27. Wash in 4 changes of PBS containing 0.05% Tween 20 and 20% DMSO at

## Gold and Silver Staining

- room temperature for 3 min each.
28. Immerse in PBS-gelatin (PBS containing 0.1% fish gelatin) for 5 min.
  29. Incubate the sections with streptavidin–Nanogold diluted 1:750 in PBS containing 1% BSA at room temperature for 60 min.
  30. Wash in PBS-gelatin for 5 min (3 times for 5 min each).
  31. Repeatedly wash in ultrapure water (EM-grade).
  32. Perform silver acetate autometallography (see Protocol 1).

### Protocol 4. DNA *In Situ* Hybridization with the GenPoint Kit in Combination with Streptavidin–Nanogold

#### *Background and Purpose*

The method combines the convenience of using a commercially available TSA kit (GenPoint Catalyzed Signal Amplification [CSA] System for *In situ* Hybridization) with the superior quality of Nanogold silver–gold detection and has been used for biotinylated or FITC-conjugated probes in DNA or RNA ISH.

#### *Solutions*

In addition to some of the solutions given in the above protocols:

- Preparation of Tris-buffered saline with Tween (TBST) (10× concentrated): 3.029 g Tris, 17.532 g sodium chloride, 5 mL Tween 20. Make up to 500 mL with double-distilled water and adjust pH to 7.6.

#### *Procedure*

1. Deparaffinize sections from formaldehyde-fixed tissue in fresh xylene (2 times for 15 min each).
2. Rinse in absolute ethanol (2 times for

5 min each), then 95% ethanol (2 times for 5 min each), followed by 2 changes of double-distilled water.

3. Immerse the slides in Target Retrieval solution (Cat. No. S1700; DAKO) at 95°C for 40 min, then let the slides cool in the same solution for 20 min.

4. Rinse the slides in several changes of double-distilled water, then incubate them with proteinase K diluted 1:5000 in 50 mM Tris-HCl buffer (pH 7.6) for 5 min at room temperature.

Alternatively, steps 3 and 4 may be replaced by pretreatment with 0.1 mg/mL proteinase K in 50 mM Tris-HCl buffer (pH 7.6) at 37°C for about 8 min (optimal duration should be tested carefully).

5. Wash slides in double-distilled water (3 changes for 5 min each).

6. Treat with 3% H<sub>2</sub>O<sub>2</sub> in methanol at room temperature for 30 min.

7. Wash slides in double-distilled water for 10 min.

8. Put slides into Lugol's iodine solution for 5 min, then wash in double-distilled water.

9. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless, then wash in double-distilled water (2 times for 5 min each).

10. Allow slides to air-dry.

11. Add one drop of biotinylated DNA probe on the section and cover with a small glass coverslip. Avoid air bubbles.

12. Heat sections on heating block at 92° to 94°C for 5 min to denature DNA.

13. Incubate in a moist chamber at 37°C overnight (or for at least 1 h).

14. Remove the coverslip by soaking slides in a TBST bath for 5 min.

15. Incubate slides in Stringent Wash (provided in the GenPoint kit) for 20 min at 55°C.

16. Drain off section, wipe area around the section dry, and surround it with a DAKO-pen.
17. Immerse slides in TBST for 5 min.
18. Apply primary streptavidin-horseradish peroxidase (HRP), diluted 1:800 in the diluent (from GenPoint kit) to sections and incubate in a moist chamber for 15 min at room temperature.
19. Wash in 3 changes of TBST for 5 min each.
20. Apply ready-to-use biotinyl-tyramide solution (from GenPoint kit) and incubate in a moist chamber for 15 min at room temperature.
21. Wash in 3 changes of TBST-gelatin (TBST containing 0.1% fish gelatin, pH 7.6) for 5 min each.
22. Incubate the sections with streptavidin-Nanogold diluted 1:250 in PBS containing 1% BSA at room temperature for 60 min.
23. Wash in 3 changes of TBST-gelatin for 5 min each.
24. Repeatedly wash in ultrapure water (EM-grade).
25. Perform autometallography (See Protocol 1).
26. After autometallographic amplification, sections can be counterstained with hematoxylin and eosin and/or nuclear fast red, dehydrated, and mounted in Permunt or in DPX.

## Protocol 5. CODFISH

### *Background and Purpose*

This protocol was developed as a response to the documented problems correlating Her-2/*neu* overexpressions—amplifications defined by IHC and FISH (24). CODFISH permits the simultaneous detection of cell membrane associated Her-2/*neu* oncoprotein (by IHC) and gene copy enu-

meration (by DNA FISH). The assay is especially suitable for an algorithm whereby brightfield IHC is used for screening.

About 70% of breast carcinoma cases will be overtly negative for Her-2/*neu* by IHC, so FISH is done for only 20% to 30% of cases which show 2+ or 3+ IHC staining, the groups presently requiring FISH for gene copy quantitation.

The CODFISH procedure is also provided as an illustration of future possible gold-silver-based systems using the same principles. At this point, the detection system for CODFISH is based on fluorescence. But eventual extension to a combination of autometallography and brightfield microscopy are clearly possible and are currently being pursued by our laboratory as well as others.

The CODFISH approach combines gene enumeration by FISH with semi-quantitative assessment of corresponding oncoprotein overexpression with alkaline phosphatase-based immunohistochemistry, exploiting the unique bifunctional properties of fast red K. This chromogen provides a bright pink-red color reaction by conventional optical microscopy, and when viewed with a rhodamine excitation-range wavelength reveals a brilliant red non-quenching fluorescence. A combination of FITC and fast red K in the fluorescence mode provides excellent visualization of the encoded protein and amplification of the gene (Figure 9.3).

This approach may have particular value in an algorithmic system where false positives may complicate interpretation. This appears to be especially true for the Her-2/*neu* oncogene system. IHC false positives are a serious complication of the laboratory assessment for eligibility for Herceptin® (Trastuzumab; Genentech, San Francisco, CA, USA) therapy. Even greater utility will be achieved when this system becomes adaptable to complete bright field visualization.

## Gold and Silver Staining

### Solutions

- Target retrieval solution.
- CB11 monoclonal antibody to Her-2/*neu* (Ventana Medical Systems/Vector Laboratories).
- Red alkaline phosphatase detection kit (Ventana Medical Systems).
- Her-2/*neu* Dig FISH kit (Ventana Medical Systems).
- Wash solutions.

### Procedure

1. Unstained paraffin sections on electrostatically charged slides are deparaffinized and rehydrated in 3 changes each of xylene, absolute alcohol, and 95% and 80% alcohol, and then placed in 1× PBS for 5 min.
2. Cell conditioning is achieved through the use of microwaving in citrate buffer for 15 min, followed by cooling of the slides in the citrate solution at room temperature for 20 min.
3. The monoclonal antibody CB11 at manufacturer's concentration is dispensed onto the sections and allowed to incubate for 1 h at room temperature.
4. Sections are then washed in 1× PBS, 2 changes of 3 min each, and biotinylated antimouse IgG (Ventana Medical Systems) applied at a concentration of 15 µg/mL for 30 min.
5. Two changes of 1× PBS wash at room temperature are followed by the addition of avidin-alkaline phosphatase (Ventana Medical Systems) at a concentration of 3000 µg/mL for 30 min.
6. Sections are then washed twice for 3 min each, in 1× PBS at room temperature, and then the chromogen reaction product developed from the fast red K proprietary solution (Ventana Medical Systems), 10 min at room temperature.
7. Sections are then washed briefly in distilled water and counterstained with hemotoxylin for approximately 30 sec. Sections are then dehydrated in graded alcohols.
8. Ten microliters of digoxigenin-labeled probe in proprietary probe solution (Oncor [Gaithersburg, MD, USA]/Ventana Medical Systems) are applied to the section and covered with a coverslip.
9. The probe solution and target tissue are codenatured at 90°C for 6 min followed by overnight hybridization at 37°C in a humidified chamber.
10. The coverslip is removed by soaking the slides in 2× SSC for 5 min at room temperature.
11. Washes of 0.5× SSC for 5 min at 72°C follow.
12. Slides are prewashed in 1× PBS containing 0.5% Tween 20 for 3 min at room temperature.
13. FITC-antidigoxigenin 1:50 in 1× PBS, pH 7.6, containing 0.5% BSA is applied to the section and allowed to incubate for 1 h.
14. The sections are then washed 3 times for 5 min each in 1× PBS containing 0.5% Tween 20.
15. The sections are then counterstained with 20 µL 4'6-diamidine-2-phenylindole (DAPI) in antifade solution (Oncor) and mounted for FISH analysis.
16. The fast red K bright red chromogenic reaction product of alkaline phosphatase also displays brilliant pink-red fluorescence on excitation using rhodamine filters. The CODFISH Her-2/*neu* copy number in 20 cells is counted in two representative fields and averaged.

**Note:** Digoxigenin as the reporter molecule may present problems when used with colloidal or clustered gold. Instead of

digoxigenin, it is better to use biotin, FITC, or other molecules in such combinations.

### Protocol 6. GOLDFISH: Auto-metallographic Subtractive Unique Sequence *In Situ* Hybridization

#### *Background and Purpose*

The GOLDFISH protocol was developed as a response to the documented problems correlating Her-2/*neu* overexpressions—amplifications defined by IHC and FISH. GOLDFISH offers a potential advantage over CODFISH and simple IHC for Her-2/*neu* protein detection. The evaluation is all done by conventional brightfield microscopy; no special equipment is required. Also, there is potential for a brightfield 2-color systems analogous to CODFISH, by the addition of alkaline phosphatase-based IHC employing fast red K as the chromogen with or without FluoroNanogold® (Nanoprobes). Such 2-color brightfield applications are currently being developed in our and others' laboratories.

This procedure outlines the system whereby single copies of the endogenous oncogene Her-2/*neu* can be detected by conventional optical microscopy. Autometallography is especially useful in this instance, since the very discrete gold–silver detection products are sharply defined as compared to enzymatic chromogenic peroxide and alkaline phosphate detection systems.

Our initial experiment focused upon the use of tyramide conjugates for this purpose. Unfortunately, the system was not readily adaptable for this particular gene, since unexpectedly high levels of background and excessive confluence of detection products were observed. Current investigation is focused upon combining this autometallographic procedure with IHC in a 2-color

system whereby protein and gene copy amplification can be simultaneously assessed with the CODFISH method.

#### *Solutions*

- Target retrieval solution.
- Her-2/*neu* Dig FISH kit (Zymed Laboratories, South San Francisco, CA, USA).
- PBS wash solution.
- Goat antimouse IgG–Nanogold.
- Autometallography solutions (Nanoprobes or as in Protocol 1).

#### *Protocol*

1. Unstained paraffin sections on electrostatically charged slides are deparaffinized and rehydrated in 3 changes each of xylene, absolute alcohol, and 95% alcohol and cell conditioning achieved through the use of microwaving in citrate buffer for 15 min, followed by cooling of the slides in the citrate solution at room temperature for 20 min.
2. Sections are then washed briefly in distilled water and then dehydrated in graded alcohols.
3. Ten microliters of digoxigenin-labeled subtracted unique sequence YAC-derived megabase probe in proprietary probe solution (Zymed) is applied to the section and covered with a coverslip.
4. The probe solution and target tissue are codenatured at 90°C for 6 min followed by overnight hybridization at 37°C in a humidified chamber.
5. The coverslip is removed by soaking the slides in 2× SSC for 5 min at room temperature.
6. Washes of 0.5× SSC for 5 min at 72°C follow.
7. Slides are prewashed in 1× PBS con-

- aining 0.5% Tween 20 for 3 min at room temperature.
8. FITC-antidigoxigenin 1:50 in 1× BCS containing 0.5% BSA is applied to the section, per slide, and allowed to incubate for 1 h.
  9. The sections are then washed 3 times for 5 min each in 1× PBS containing 0.5% Tween 20.
  10. The sections are then overlaid with mouse anti-FITC (Roche Molecular Biochemicals) at 1:800 dilution in PBS with 0.5% Tween 20.
  11. Following PBS washing, 3 times for 5 min each in 1× PBS containing 0.5% Tween, biotinylated goat antimouse IgG-Nanogold at 1:200 is applied for 30 min with intervening PBS washes.
  12. Autometallography is performed with silver acetate or lactate developing solution for 8 min, and the reaction is stopped by immersion in distilled water.
  13. The slides are then dehydrated in graded alcohols and xylene and covered with a coverslip.
  14. Her-2/*neu* copy number is displayed as black spherical nuclear granules (Figure 9.4, A and B).

### DISCUSSION AND TECHNICAL HINTS

The explosive growth of knowledge of the human genome, the greatly improved access to large-scale screening via tissue microarrays, and the advent of new probe technologies such as subtractive, unique sequence ISH probes derived from BACs or YACs that dramatically augment signal intensity by virtue of probe size and sequence specificity, are revolutionizing the technique of ISH.<sup>1,3,19,20</sup> Even chromosomal deletions can be profiled in interphase FISH or CISH format, opening even

greater vistas for the revised and rejuvenated procedure, ISH.

The purpose of this chapter is not to review the technique of ISH in technical detail. There are several excellent reviews of the method extant in the published literature. Only the highlights of particular importance to the autometallographic modifications of ISH are noted here.

The value of appropriate cell conditioning in accounting for the remarkable improvements in ISH results cannot be overemphasized. Microwaving, simple heat retrieval, steam systems, pressure cookers, and simple water bath heat conditioning, have all improved the ability to see fewer copies of DNA/RNA targets with a little or no background staining. Automation of ISH by the newly manufactured Discovery in the Nexus Plus staining robot accomplishes all of these steps, also including deparaffinization and counterstaining, directly on-line on the instruments. And these instruments also allow for separate heating and buffer treatments of each of 20 separate slides, each stained by a unique protocol.

All of the general principles underlying successful ISH experiments apply to the autometallographic modifications of the procedure. For example, careful attention to probe characteristics, stringency of washing solutions, hybridization conditions, and accuracy of denaturalization temperatures and conditions, must all be rigorously controlled and carefully monitored for successful staining, just as with FISH and chromogenic ISH systems. Hybridization requirements vary for probes in all systems; generally reflecting melting temperatures as a consequence of a GC base ratios. The formula

$$T_m - 5^\circ\text{C} = 0.1 \times \text{SSC} @ 60^\circ\text{C}$$

can be used as general guideline for determination of the appropriate melting temperature of the probe, but in our experi-



ence, the formula is only useful for "ball-parking" experimental conditions—nearly always, additional empiric experiments are required to find the "sweet spot."

When using tyramide systems, the contribution of endogenous peroxidase cannot be overemphasized. Even the smallest amount of peroxidase or pseudoperoxidase will result in catalysis of the tyramide conjugate and will seriously contribute to background staining.<sup>25</sup> When using oligonucleotides directly conjugated to peroxidase, it is critical that one modifies the system so that the peroxidase activity of the conjugate is preserved. Temperatures above approximately 52°C will destroy peroxidase activity from the very expensive probe reagent.

Concerning reporter molecules and probe size, we have extensively tested biotin-labeled cDNA probes for the detection of viral DNA. In these tests, we noted that some of the probes gave excellent results and in most cases were ISH stained using Protocols 1 through 4 (e.g., HPV 16/18; Enzo). However, we also noted that some other biotinylated cDNA probes (e.g., HPV 31/33/51; Enzo) most often lead to unacceptable background staining when used in combination with labeled tyramides, thus making efficient reading of the sections impossible. In case of RNA, we have successfully worked with self-made FITC-labeled riboprobes (Protocol 3). Using digoxigenin as the probe label, in many experiments, successful staining was never obtained when the TSA system was applied. The reason for this incompatibility is not yet clarified, but may be explained by steric hindrance. Probe size is also an important consideration. Whereas cDNAs and riboprobes give excellent sensitivity in many situations, oligonucleotides have not yet been extensively tested by us. Earlier, only some 20-bp long oligonucleotides could be made and labeled by one or two biotin molecules only. However, today it is relatively easy to synthesize oligoprobes of

much larger size, and methods have been proposed that allow a multiple labeling with reporter molecules (e.g., using the LabelIT nucleic acid labeling kits; Cat. No. MIR3400; Mirus, Madison, WI, USA). Such systems should make it possible now to detect single copies of genes or message by TSA by custom synthesis probes, which can then be optimally adapted for each specific application.

For the autometallographic development, whether gold-enhanced or silver acetate-lactate solutions are used (see Chapters 2 and 3), it is best to first optimize staining by monitoring with conventional optical microscopy. But eventually, for assurance of reproducible performance of the procedure, conditions should be finalized in such a way that a standard time for a silver development can be identified and followed. It is imperative that sodium thiosulfate not be used to stop the silver reactions, as this reagent will most often destroy the visualization product that has been developed. On the other hand, extraction of endogenous trace metals with Lugol's iodine solution and bleaching with thiosulfate early in the ISH procedure is absolutely imperative for proper performance of autometallographic ISH.<sup>7,23,25</sup>

Acknowledgement: U.S.D.O.E., NIH

## REFERENCES

1. Bubendorf, L., J. Kononen, P. Koivisto, P. Schraml, H. Moch, T.C. Gasser, N. Willi, M.J. Mihatsch, G. Sauter, and O.P. Kallioniemi. 1999. Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence *in situ* hybridization on tissue microarrays. *Cancer Res.* 59:803-806.
2. Cheung, A.L.M., A.H. Graf, C. Hauser-Kronberger, O. Dietze, R. Tubbs, and G.W. Hacker. 1999. Detection of human papillomavirus in cervical carcinoma: comparison of peroxidase, Nanogold, and catalyzed reporter deposition (CARD)-Nanogold *in situ* hybridization. *Mod. Pathol.* 12:689-696.
3. Davidson, J.M., T.W. Morgan, B.L. Hsi, S. Xiao, and J.A. Fletcher. 1998. Subtracted, unique-sequence *in situ* hybridization. *Am. J. Pathol.* 153:1401-1409.
4. Danscher, G., G.W. Hacker, J.O. Norgaard, and L. Grimelius. 1993. Autometallographic silver amplification of colloid gold. *J. Histochemol.* 16:201-207.

5. Danscher, G., G.W. Hacker, C. Hauser-Kronberger, and L. Grimelius. 1995. Trends in autometallographic silver amplification of colloidal gold particles, p. 11-18. In M.A. Hayat (Ed.), *Immunogold-Silver Staining: Methods and Applications*. CRC Press, Boca Raton.
6. De Bault, L.E. and B.L. Wang. 1994. Localization of mRNA by *in situ* transcription and immunogold-silver staining. *Cell Vision* 1:67-70.
7. Fujimori, O., T. Ueda, and K. Yamada. 1994. Effects of iodine pretreatment of sections upon immunogold-silver staining in light microscopic immunohistochemistry. *Okajimas Folia Anat. Jpn.* 71:319-323.
8. Graf, A.-H., A.L.M. Cheung, C. Hauser-Kronberger, N. Dandachi, R.R. Tubbs, O. Dietze, and G.W. Hacker. 2000. Clinical relevance of HPV 16/18 testing methods in cervical squamous cell carcinoma. *Appl. Immunohist. Mol. Morphol. (AIMM)* (In press).
9. Hacker, G.W., L. Grimelius, G. Danscher, G. Bernatzky, W. Muss, H. Adam, and J. Thurner. 1988. Silver acetate autometallography: an alternative enhancement technique for immunogold-silver staining (IGSS) and silver amplification of gold, silver, mercury, and zinc tissues. *J. Histotechnol.* 11:213-221.
10. Hacker, G.W., A.-H. Graf, C. Hauser-Kronberger, G. Wirnsberger, A. Schiechl, G. Bernatzky, U. Wittauer, H. Su, H. Adam, J. Thurner, et al. 1993. Application of silver acetate autometallography and gold-silver staining methods for *in situ* DNA hybridization. *Chin. Med. J.* 106:83-92.
11. Hacker, G.W. and G. Danscher. 1994. Recent advances immunogold-silver staining-autometallography. *Cell Vision* 1:102-109.
12. Hacker, G.W., G. Danscher, L. Grimelius, C. Hauser-Kronberger, W. H. Muss, J. Gu, and O. Dietze. 1995. Silver staining techniques, with special reference to the use of different silver salts in light and electron microscopical immunogold-silver staining, p. 20-45. In M.A. Hayat (Ed.), *Immunogold-Silver Staining: Methods and Applications*. CRC Press, Boca Raton.
13. Hacker, G.W., I. Zehbe, J. Hainfeld, J. Sallstrom, C. Hauser-Kronberger, A.-H. Graf, H. Su, O. Dietze, and O. Bagasra. 1996. High performance Nanogold *in situ* hybridization and *in situ* PCR. *Cell Vision* 3:209-214.
14. Hacker, G.W. and G. Danscher. 1997. Immunogold-silver staining-autometallography: recent developments and protocols, p. 41-54. In J. Gu (Ed.), *Analytical Morphology—Theory, Protocols, and Applications*. Eaton Publishing, Natick, MA.
15. Hacker, G.W., C. Hauser-Kronberger, I. Zehbe, H. Su, A. Schiechl, O. Dietze, and R. Tubbs. 1997. *In situ* localization of DNA and RNA sequences: super-sensitive *in situ* hybridization using streptavidin-Nanogold silver staining: mini review, protocols, and possible applications. *Cell Vision* 4:54-67.
16. Hacker, G.W. 1998. High-performance Nanogold-silver *in situ* hybridisation. *Eur. J. Histochem.* 42:111-120.
17. Hainfeld, J.F. 1987. A small gold-conjugated antibody label: improved resolution for electron microscopy. *Science* 236:450-453.
18. Hainfeld, J.F. and E.R. Furuya. 1992. A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J. Histochem. Cytochem.* 30:177-184.
19. Kononen, J., L. Bubendorf, A. Kallioniemi, M. Barlund, P. Schraml, S. Leighton, J. Torhorst, M.J. Mihatsch, G. Sauter, and O.P. Kallioniemi. 1998. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.* 4:844-847.
20. Moch, H., P. Schraml, L. Bubendorf, M. Mirlacher, J. Kononen, T. Gasser, M.J. Mihatsch, O.P. Kallioniemi, and G. Sauter. 1999. High-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarray screening in renal cell carcinoma. *Am. J. Pathol.* 154:981-986.
21. Powell, R.D., C.M.R. Halsey, D.L. Spector, S.L. Kaurin, J. McCann, and J.F. Hainfeld. 1997. A covalent fluorescent-gold immunoprobe: simultaneous detection of a pre-mRNA splicing factor by light and electron microscopy. *J. Histochem. Cytochem.* 45:947-956.
22. Rufner, R., N.E. Carson, M. Forte, G. Danscher, J. Gu, and G.W. Hacker. 1995. Autometallography for immunogold-silver staining in light and electron microscopy. *Cell Vision* 2:327-333.
23. Tbakhi, A. G. Totos, C. Hauser-Kronberger, J. Pettay, D. Baunoch, G.W. Hacker, and R.R. Tubbs. 1998. Fixation conditions for DNA and RNA *in situ* hybridization: a reassessment of molecular morphology dogma. *Am. J. Pathol.* 152:35-41.
24. Tubbs, R.R., J. Pettay, T. Grogan, M. Stoler, and P. Roche. 2000. CODFISH (concomitant oncoprotein detection and fluorescence *in situ* hybridization). *J. Mol. Diag.* 2:78-83.
25. Zehbe I., G.W. Hacker, H. Su, C. Hauser-Kronberger, J.F. Hainfeld, and R.R. Tubbs. 1997. Sensitive *in situ* hybridization with catalyzed reporter deposition, streptavidin-Nanogold, and silver acetate autometallography: detection of single-copy human papillomavirus. *Am. J. Pathol.* 150:1553-1561.